Identification and Assessment of Endocrine Disruptors: Limitations of *in Vivo* and *in Vitro* Assays

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It has been suggested that chemicals and complex mixtures capable of modulating the endocrine system may contribute to adverse health, reproduction, and developmental effects in humans and wildlife. These effects include increased incidence of hormone-dependent cancers, compromised reproductive fitness, and abnormal reproductive system development. In response to public concern, regulatory agencies in North America and Europe are formulating potential strategies to systematically test chemicals and complex mixtures for their endocrine-disrupting activities. Because of the complexity of the endocrine system and the number of potential endocrine disruptor targets, a tiered approach involving a complementary battery of short- and long-term *in vivo* and *in vitro* assays that assesses both receptor and nonreceptor-mediated mechanisms of action is being considered. However, the available established assays use a limited number of end points, and significant information gaps exist for other potential targets in the endocrine system. In addition to discussing the merits and limitations of the assays that may be adopted, this paper also highlights potential problems associated with the use of a tiered testing strategy. — *Environ Health Perspect* 106(Suppl 2):577–582 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/577-582zacharewski/abstract.html*

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Introduction

Epidemiologic studies have found significant increases in the incidence of hormone-dependent diseases including cancers of the breast, prostate, and testis (*1–8*). For example, the U.S. National Cancer Institute's Surveillance Epidemiology and End Results (SEER) Program reports that newly diagnosed cases of breast cancer increased at an annual rate of 1% between 1950 and 1979. This diagnosis rate tripled to 3% per year from 1980 to 1984 (*9*). Glass and Hoover have shown that the largest increases in incidence have occurred in women 60 years of age and older (74%), and in those 45 to 59 years of age (36%) (*10*). In women between 20 to 44 years of age, the rate has remained essentially unchanged. Less than one-third of the overall 15.3% increase in the age-adjusted rate for invasive breast cancers seen between 1972 and 1985 could be attributed to the increased use of screening mammography (*5*).

The occurrence of breast cancer has been associated with affluent societies, and studies have shown that the rates of occurrence can vary by as much as 5- to 10-fold between countries. Moreover, migrants who move from low- to high-risk countries adopt the rates of their new country. There have also been reports of significant increases in the incidence of male reproductive tract disorders (e.g., maldescent [cryptorchidism], urethal abnormalities [hypospadias]), decreases in semen volume and sperm counts, and compromised reproductive fitness in humans and wildlife (*7,11–14*). These results suggest that environmental factors may contribute to the increased incidence of these adverse effects (*15,16*).

This hypothesis is supported by several paradigms that have shown that the development of cancers and the occurrence of reproductive tract disorders can be influenced by exposure to estrogens or estrogenic drugs. These include

- experimental studies demonstrating the ability of sex steroids to promote tumor development (*17–20*);
- epidemiologic studies reporting the protective effect of ovariectomization, the increased risk of breast cancer in young women exposed to diethylstilbestrol, and the association between maternal estrogen concentrations and the frequency of testicular cancer and cryptorchidism (*19,21–23*);
- the prevalence of infertility and malformations of the genitalia in male rodents exposed prenatally to diethylstilbestrol (*24,25*); and
- the efficacy of hormone antagonists in treating cancers (*26,27*).

Consequently, it has been suggested that xenobiotics capable of mimicking or blocking the activities of sex steroids may play a role in the etiology of hormone-dependent cancers and disorders of the male reproductive tract in humans and wildlife (*12,13,15,28–35*).

Exogenous substances that can elicit sex steroidlike activities are commonly referred to as endocrine disruptors and have been defined as any exogenous agent, either synthetic or natural, that interferes with the production, release, transport, metabolism, binding, biologic action, or elimination of natural ligands in the body that are responsible for the maintenance of homeostasis and the regulation of developmental processes. In many cases, these endocrine disruptors share no apparent structural similarities to traditional steroids. Endocrine disruptors include natural products (phytoestrogens, e.g., genistein) (*36–38*), pharmaceuticals (i.e., diethylstilbestrol, ethynyl estradiol) (*39*), environmental pollutants (i.e., DDT, polychlorinated biphenyls, dioxins, polyaromatic hydrocarbons) (*40–44*), and industrially relevant chemicals (i.e., alkylphenols, bisphenol A) (*45–47*). The potential exposure and economic significance of several of these substances have made endocrine-disrupting chemicals a contentious health concern and environmental issue.

In contrast, several studies suggest that endocrine disruptors may not significantly

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Abbreviations used: E2, 17β-estradiol; ER, estrogen receptor; QSAR, quantitative structure–activity relationship; SHBG, sex hormone-binding globulin.

contribute to the development of hormone-dependent disease or compromise reproductive fitness in humans. For example, Wilcox et al. (*48*) report that men who were prenatally exposed to diethylstilbestrol experienced no impairment of fertility based on the following parameters: incidence of impregnation, age and birth of first child, average number of children, diagnosis of fertility problems, and length of time to conception. In addition, no impairment of sexual function as indicated by the frequency of intercourse or reported episodes of decreased libido were reported (*48*). Consistent with the results of this study, several recent reports provide evidence refuting the findings that sperm counts and semen quality have been decreasing (*49–51*). Furthermore, women on vegetarian diets who consume large quantities of natural products with weak estrogenic activities (e.g., phytoestrogens) have a lower incidence of breast cancer (*52,53*). This evidence has led some researchers to suggest that weak estrogen agonists may function as antiestrogens in the presence of potent agonists, and therefore, do not contribute to the development of hormone-dependent diseases and developmental abnormalities and may even serve a protective role in some situations (*54–56*).

In Vivo Assessment of Endocrine Disruptors

As suggested by the endocrine disruptor definition, there are a number of potential mechanisms of action that may be susceptible to the adverse effects of endocrine disruptors. This diversity of potential targets and the complexity of feedback mechanisms significantly complicates evaluating the consequences of exposure to endocrine disruptors. Fortunately, many of the functions and mechanisms of the endocrine system are conserved among species, thereby providing scientists methodologies, initially developed for clinical medicine and drug discovery or evaluation, to investigate the activities of endocrine disruptors. A compendium listing the assays currently used to test for the estrogenic activities of a substance or complex mixture has recently been compiled (*57*). Several of these tests (e.g., enzyme induction, cell differentiation, effects on organ weights) take advantage of the receptor-mediated mechanism of action of sex steroids. The advantages and disadvantages of some of these *in vitro* and *in vivo* assays as well as emerging methodologies have recently been reviewed (*58,59*). Endocrine disruptors also elicit effects through receptor-independent mechanisms that may involve steroid transport (i.e., hormone-binding globulins) (*60,61*), steroid synthesis (i.e., inhibition of aromatase activity) (*62*), or interactions with target cell membranes (*63,64*). Therefore, a comprehensive evaluation of an endocrine disruptor requires a battery of complementary *in vitro* and *in vivo* assays that are based on receptor- and nonreceptor-mediated mechanisms.

There are a limited number of short-term, established *in vivo* assays that could be used to assess endocrine disruptors. However, it is questionable whether these assays alone can accurately identify and assess chemicals, natural products, environmental pollutants, and complex mixtures alleged to possess endocrine-disrupting activities. For example, two classical assays, namely the uterotrophic and vaginal cell cornification assays, are the most widely used *in vivo* assays for assessing estrogenic substances (*58,65–70*). Increases in uterine wet weight is an established measure of the estrogenicity of a compound and is the hallmark for the definition of an estrogen or the identification of an estrogenic substance (*66,71*). Previous studies examining the effects of estrogenic substances on uterine wet weight have used a number of different protocols and species; therefore, uterotrophic assays require standardized operating procedures that specify species, strain, age, and route of test compound administration in addition to other potential interventions (i.e., ovariectomy). For example, it has been reported that there are marked species differences in responsiveness and that the mouse uterus is much more sensitive to estrogens than the rat (*66*). In addition, although studies suggest that the uterotrophic assay exhibits greater sensitivity in immature, ovariectomized animals (*69,72*), the uterus also responds to progesterone, testosterone, and other agents that are not characteristically estrogenic, which can lead to confounding results (*73–77*).

In contrast, vaginal epithelial cell cornification in ovariectomized rodents can be induced only by compounds considered to be estrogenic. It is believed to be a definitive *in vivo* test for identifying estrogenic substances or complex mixtures (*78*). Although the assay has the advantage of being relatively simple and can use the same animals repeatedly provided the test compound does not bioaccumulate, it has been criticized as being largely qualitative as scoring is dependent on the evaluation of cellular contents of a vaginal lavage. In addition, the assay requires large numbers of animals to ensure accurate results (*66,68*). The qualitative nature of the assay has been somewhat addressed by introducing a grading system that involves scoring the degree of cornification using the disappearance of leukocytes and the appearance of cornified squamous cells (*79*).

There has also been some concern that short-term rodent assays may not possess sufficient sensitivity to identify substances and complex mixtures with weak or specific endocrine-disrupting activities. It is conceivable that endocrine disruptors may elicit responses at the gene expression level that may not be translated into immediate responses at the organ or tissue level but could subsequently predispose an individual or subpopulation to adverse effects at later stages of development. Assessment of endocrine disruptors is further complicated by the fact that many substances elicit species-, tissue-, cell-, and response-specific effects. For example, some estrogens are more effective for imbibition of uterine fluid, whereas others are more active in the promotion of uterine growth. Moreover, superior efficacy for one response does not indicate that the same rank order of potency will be exhibited for a different response (*66*). Another example is tamoxifen, which exhibits antiestrogenic activity in the breast and agonist activities in the uterus. These examples demonstrate the necessity of measuring a number of different end points in order to comprehensively evaluate the potential endocrine-disrupting activities of a substance or complex mixture.

The appropriateness of using rodents as models to assess the risk that endocrine disruptors pose to human and wildlife health has also been questioned as rodents do not express sex hormone-binding globulin (SHBG) following parturition. SHBG is a 17β-estradiol-inducible circulating serum protein that exhibits significant changes in expression levels during development in all vertebrate species and is a major determinant of the metabolic clearance and the bioavailability of sex steroids (*60,80,81*). In addition, specific receptors for SHBG and ligand-bound SHBG have been identified in prostatic, placental, endometrial, and breast cells (*82–91*) that may be involved in a cAMP-dependent signaling pathway that induces cell growth (*92–95*). Intracellular SHBG has also been identified in these tissues, suggesting a physiologic function for this protein in cellular sex steroid uptake (*88,90*). Although it is unclear if endocrine disruptor interaction

with SHBG plays a role in eliciting adverse effects, it is known that humans and some wildlife species express SHBG after parturition. Therefore, SHBG may be a potential target or protective measure against endocrine disruption that to date has not been adequately considered.

In Vitro Assays for Endocrine Disruptors

A number of *in vitro* assays are also based on known mechanisms of action of sex steroids. These include

- measuring the activity of enzymes involved in steroid synthesis (*62,96,97*);
- competitive ligand binding assays using binding globulins (*61,98,99*) and receptors (*43,59,100*);
- cell proliferation assays (*101–104*); and
- gene expression assays in mammalian cells and yeast (*59,105–110*).

However, many of these assays lack standardized operating procedures with suggested performance guidelines based on appropriate controls and proficiency samples. This is critical as *in vitro* assay performance can be fickle because of differences in media formulations, serum source, and cell line strains (*59,111*).

In addition, the ability to predict responses *in vivo* is questionable as it is not possible to accurately reproduce the *in vivo* pharmacokinetic and pharmacodynamic interactions in *in vitro* assays. For example, *in vitro* assays do not possess the same metabolic capabilities present *in vivo* and therefore may generate false positive results due to the inability to metabolically inactivate an estrogenic substance. This has been observed with selected phthalate esters that were found to induce weak estrogen receptor-mediated effects *in vitro* (*112,113*) but did not elicit a response *in vivo,* as evidenced by uterotrophic and vaginal cornification assays (*113*). Potentially more problematic are false negative results that are due to the inability of *in vitro* systems to bioactivate a proestrogen to its estrogenic metabolite. However, several *in vitro* systems possess some metabolic capabilities and, to date, there have been no reported examples of *in vitro* assays generating false negative results even with endocrine disruptors that are known to require bioactivation (i.e., methoxychlor, polychlorinated biphenyls) (*41*).

Summary

To comprehensively assess the potential endocrine disrupting activities of a substance or complex mixture, it is essential that a complementary battery of *in vitro* and *in vivo* assays be used. This battery could involve a tiered strategy consisting of computational models such as quantitative structure–activity relationship (QSAR), paradigms, *in vitro* assays and short-term *in vivo* assays in tier I, longer term *in vivo* assays in tier II, and if necessary, mulitigenerational studies in tier III. In this scheme, subsequent tier testing would be triggered following a review of the results obtained in the preceding tier. Uncertainties in this strategy arise when determining what constitutes sufficient data to warrant further testing. For example, there is no doubt that endocrine disruption in short-term *in vivo* studies in tier I would provide sufficient evidence to warrant further testing in tier II. However, the course of action may be less clear when there is a lack of an effect *in vivo,* but a response is observed using *in vitro* assays as well as positive predictions of endocrine-disrupting activities from QSAR models. Therefore, it may be prudent to establish guidelines that outline criteria which essentially exculpate a chemical or complex mixture that is suspected of eliciting endocrine-disrupting activities, in order to avoid a futile testing loop.

It is also clear that further research is required for the development of new *in vitro* and *in vivo* assays. Currently, there are inadequate *in vitro* and *in vivo* testing methodologies for several known potential targets such as the thyroid and androgen receptor systems. Moreover, there is a complete lack of knowledge regarding the impact of endocrine disruptors on other potential endocrine targets and mechanisms of action, including crosstalk between membrane-bound and nuclear receptors (*114–117*), the roles of new (*118,119*) and orphan receptors (*120–122*), and the effect on growth factor-mediated signal transduction. Needless to say, prior to the incorporation of any of these assays into a screening protocol, they should be subjected to a rigorous evaluation to determine their advantages and limitations, as well as to define how this information will be used in risk assessment and regulatory arenas.

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